A comprehensive study on the role of the *Yersinia pestis* virulence markers in an animal model of pneumonic plague

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Abstract We determined the role of *Yersinia pestis* virulence markers in an animal model of pneumonic plague. Eleven strains of *Y. pestis* were characterized using PCR assays to detect the presence of known virulence genes both encoded by the three plasmids as well as chromosomal markers. The virulence of all *Y. pestis* strains was compared in a mouse model for pneumonic plague.

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The presence of all known virulence genes correlated completely with virulence in the Balb/c mouse model. Strains which lacked HmsF initially exhibited visible signs of disease whereas all other strains (except wild-type strains) did not exhibit any disease signs. Forty-eight hours post-infection, mice which had received HmsF⁻ strains regained body mass and were able to control infection; those infected with strains possessing a full complement of virulence genes suffered from fatal disease. The bacterial loads observed in the lung and other tissues reflected the observed clinical signs as did the cytokine changes measured in these animals. We can conclude that all known virulence genes are required for the establishment of pneumonic plague in mammalian animal models, the role of HmsF being of particular importance in disease progression.

Yersinia pestis, a Gram-negative bacillus is the causative agent of the disease plague which is a zoonotic disease primarily affecting rodents; the human host plays no part in the long term survival of *Y. pestis*. Transmission between rodents occurs via fleas, *Y. pestis* being acquired from an infected blood meal, infection in these hosts being confined to the alimentary canal. Transfer to other rodents occurs via regurgitation of the bacteria as the fleas repeatedly try to feed, a consequence of infection by *Y. pestis* is the blockage of the proventricular valve thus preventing blood meals entering the stomach (Perry and Fetherston 1997). The human disease manifests itself in three different forms; bubonic and primary scepticemic plague both of which are spread by fleas, although the former has a fatality rate of 40–60% whereas the latter is 100% fatal if untreated. The third manifestation is primary pneumonic plague which is

spread by aerosol droplets and, like scepticemic plague, is 100% fatal if left untreated. Secondary pneumonic plague can occur in cases of bubonic plague that remain untreated (Prentice and Rahalison 2007).

A number of plasmid and chromosome-encoded virulence genes have been identified in *Y. pestis* including those involved in Congo red binding (Crb$^+$ phenotype), which forms the basis of an assay which is used as an indicator of the presence of a number of virulence genes collectively involved in iron uptake, and the pigmentation phenotype (Pgm$^+$ phenotype) encoded by a 102-kb locus (*pgm* locus) part of which encodes a siderophore-dependent iron transport system (Perry et al. 2004). PGM$^-$ bacteria are avirulent in the mouse model unless infection occurs via the intravenous route or bacteria are supplemented with an exogenous source of iron (Staggs et al. 1994). Other important virulence factors are the proteins pesticin, plasminogen activator (pPCP1; 9.5 kb), and the F1 (pMT1; 110 kb) and V-antigens (pCD1; 75 kb). All of these virulence factors are plasmid encoded as indicated, and loss of any of these plasmids variably affects virulence in animal models dependent upon the route of infection (Zauberman et al. 2009). Indeed, in wild-type strains, growth at 25°C compared with 37°C was found to significantly increase the LD$_{50}$ when mice were infected via the aerosol route, however, other routes of infection were unaffected (Perry and Fetherston 1997). The kinetics of pneumonic plague development have recently been characterized and described in both the mouse as well as in the brown Norwegian rat model (Agar et al. 2008; Anderson et al. 2009; Agar et al. 2009). In these model systems, the fully virulent strain CO92 was used as the challenge strain and similar LD$_{50}$'s as well as times to death were observed in both animal models. In these models, it was postulated that the observed early pro-inflammatory response was induced by the type III secretion system (encoded by the pCD1 plasmid) and its associated effectors, whilst the latter pro-inflammatory response resulted from the production of a number of cytokines and chemokines, specifically IL-1β, IL-1α, IFN-γ, IL-12, and IL-6 (Agar et al. 2008).

There are numerous strains of *Y. pestis* residing in culture collections throughout the world, many of which remain uncharacterized in terms of their virulence in pneumonic and bubonic plague animal models. Many molecular assays for detecting the presence of genes encoding the known key virulence determinants have also been described (Tomaso et al. 2008; Matero et al. 2009). However, although these assays exist and have been used for clinical diagnosis or environmental detection there are no reports correlating the presence of known virulence gene markers as detected by polymerase chain reaction (PCR) with virulence in animal models of pneumonic plague. This

study, therefore, characterized the strains of *Y. pestis* which were available from the *Health Protection Agencies National Collection of Type Cultures* (NCTC) in London for the presence of known virulence gene markers and correlated their virulence in the mouse model of pneumonic plague.

Material and methods

Culture of bacterial strains for molecular analysis Y. pestis strains (see Table 2) were obtained as lyophilized cultures directly from the *National Collection of Type Cultures* (NCTC), HPA (London, UK). Strains were reconstituted in 0.5-mL sterile water, aliquots plated onto Columbia Horse Blood agar plates (*bioMérieux*) and incubated at 37°C for 48 h. Cultures were checked for purity before subculture onto fresh Columbia Horse Blood agar and CMOX agar. Single colonies (six) were examined for each of the strains from both media types by polymerase chain reaction (PCR) assay (see below). Cultures grown on the previously described media were also incubated at 28°C and colonies examined using the same methodology (Tomaso et al. 2008).

Culture of bacterial strains for challenge stocks To prepare challenge stocks of *Y. pestis*, bacteria were cultured in Brain Heart Infusion (BHI) broth for 24 h at 26°C. The bacterial pellet was washed twice in sterile phosphate-buffered saline (PBS), resuspended in the same buffer and stored overnight at 4°C. The concentration was determined by plating 10-fold serial dilutions on Trypticase Soy Agar (TSA) plates. Plates were incubated at 26°C, and CFU enumerated after a 3-day incubation. Pulmonary infection with *Y. pestis* was performed by intranasal (i.n.) inoculation of 2×10^5 CFU bacteria in a 50-μL volume. Five mice were used for each *Y. pestis* strain, with another five mice used as control (non-infected). Three days post-infection mice were euthanized by an overdose of Nembutal, and the bacterial load within the organs was determined.

Polymerase chain reaction Template material for PCR was produced by thermal lysis of emulsified colonies obtained as described previously (Tomaso et al. 2008). Used primer and probe sets are listed in Table 1. Colonies were emulsified in sterile distilled water (100 μL) and heated at 100°C for 10 min. Aliquots were examined for sterility by plating onto Columbia Horse Blood agar before thermal lysates were removed from containment facilities. PCR reactions contained: 2× Fast Universal Master mix (*Applied Biosystems*) 10 μL, forward primer (18 nmol/L) 1 μL, reverse primer (18 nmol/L, 1 μL); probe (5 mmol/L) 1 μL, RNase free water 5 μL, DNA template 2 μL. Reactions

Table 1 PCR primers, probes, and loci used

Plasmid	Locus	Sequences, 3′→5′		
		Forward primer	Reverse primer	Probe
pCD1	lcrV	CGG CGG TTA AAG AGA AAT GC	CAT CGC CGA ATA CAC AAT GG	TAC TGC CAT GAA CGC C
pMT1	caF1	TTG GCG GCT ATA AAA CAGG AA	CAC CCG CGG CAT CTG TA	CAC TAG CAC ATC TGT TAA C
pPCP1	pst1	CGG CAA TCG TTC CCT CAA	GGT CAG GAA AAA GAC GGT GTG A	AAC CAT GAC ACG GTA GAC T
HmsF[a]	hmsHFRS	CGG AGA AGC CAA CGT TCG T	TCT TTC ACT TTG CGG CAA TG	CCG CCT GCA CAA CG

[a] Chromosomal

were submitted to 40 cycles (*ABI* 7500 fast Protocol) comprising 95°C, 3 s; 60°C, 30 s.

Animals Balb/c mice (*Harlan*, the Netherlands) were used. Adult (6- to 8-week-old) animals were kept in sterile isolators in a biohazard animal unit. Animals were fed irradiated food (*Harlan*) and allowed to imbibe acidified water ad libitum. The infected mice were monitored regularly for clinical status and weighed daily. All experimental procedures performed on the animals were approved by the *Ethical Committee on Animal Experimentati*on at TNO.

Determination of bacterial load in tissues of infected animals Lungs, spleens and livers of the infected animals were aseptically removed to determine the number of bacteria in these organs. Organs were homogenized in 5-mL sterile PBS using the MicroBiomaster (*Seward*, UK). The homogenates were serially diluted and plated on TSA plates. Plates were incubated at 26°C, and CFUs were determined after a 3-day incubation.

Infection of human PBMCs in vitro Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (*Sanquin Bloodbank*, Rotterdam), derived from three different volunteers, within 24 h after venepuncture by density centrifugation on Ficoll Paque Plus (*Amersham Pharmacia*). PBMCs were washed and seeded in 24-well culture plates (*Costar*) at a concentration of 10^6 cells per well in RPMI-1640 (*BioWhittaker*) supplemented with 10% FCS (*PAA*). The *Y. pestis* strains, grown in BHI medium at 26°C, were used to infect the PBMCs at a multiplicity of infection of 10. Subsequently, the PBMCs were incubated at 37°C in 5% CO_2 atmosphere. Levels of TNF-α and IL-6 were determined in the culture supernatants by enzyme-linked immunosorbent assay (ELISA; PeliKine compact; *Sanquin*) at 4 and 48 h post-infection, respectively. Cytotoxicity of the *Y. pestis* strains was determined by measuring LDH activity in the culture supernatant 48 h post-infection using the Cytotoxicity Detection kit (*Roche*).

Statistical analysis Data were analyzed for statistical significance using a two-way ANOVA for the weight curve and unpaired, one-tailed Students *t* tests for bacterial load, cytokine levels and cytotoxicity. All data are presented as mean±standard error of the mean.

Results

Molecular analysis of Y. pestis strains Currently, the link between the presence of different gene markers and virulence of *Y. pestis* isolates is rather unclear; the absolute determination of virulence requires an in vivo challenge study to be performed. Moreover, the culture collections in different countries, containing these pathogens often do not have access to such in vivo study data. We therefore designed PCR primers and probes against the known plasmid-derived virulence marker genes *LcrV*, *pst1*, and *caF1*, as well as the chromosomal locus *HmsF* (Table 1). The presence or absence of the different genetic markers was determined using real-time PCR, based upon amplification of the gene rather than detection of the mRNA (Table 2). Growth on CMOX agar plates also allowed the ability of the strains to adsorb the dye Congo red to be evaluated. For each strain there was complete correlation of the phenotypic property of Congo red adsorption with the presence of the HmsF locus, as expected. Only NCTC strains 570, 5923, and 8779 did not show the classical red-pigmented colonies when grown on CMOX agar. Pigmentation of the colonies was observed in HmsF$^+$ strains irrespective of the growth temperature or whether strains had been previously sub-cultured on alternative growth media.

Assessment of Y. pestis virulence in a mouse model of pneumonic plague To determine the importance of the role of the different *Y. pestis* virulence markers examined by PCR (HmsF, pCD1, and pMT1) in the progression of pneumonic plague, mice were intranasally infected with the *Y. pestis* strains (Table 2). After 24 h, the mice that received the *Y. pestis* strains lacking HmsF, had a ruffled fur and

Table 2 *Yersinia pestis* strains used, including Ct values from the real-time PCR analysis

NCTC number	Designation	Ct values			
		HmsF	pMT1	pCD1	pPCP1
10030	Human, fatal bubonic plague	19.05 (+)	19.40 (+)	17.67 (+)	24.85 (+)
10329	Isolated Nairobi 1963	19.47 (+)	19.76 (+)	18.01 (+)	28.50 (+)
10029	Human, bubonic plague	23.41 (+)	23.96 (+)	20.26 (+)	20.13 (+)
10330	Isolated Nairobi 1963	19.20 (+)	19.83 (+)	17.84 (+)	17.07 (+)
8775	Isolated Manchester 1953, ex India	19.83 (+)	20.73 (+)	18.83 (+)	17.97 (+)
570	Human, fatal bubonic plague, Bombay 1920	n.d. (−)	22.10 (+)	19.78 (+)	28.91 (+)
5923	Java type strain 1939	n.d. (−)	22.10 (+)	19.36 (+)	17.60 (+)
2868	Isolated Bombay 1928	20.74 (+)	21.54 (+)	n.d. (−)	22.52 (+)
5924	Isolated from Javan strain 1939	18.79 (+)	19.62 (+)	n.d. (−)	17.93 (+)
2028	Javan plague, Java 1925	n.d. (−)	23.71 (+)	n.d. (−)	18.73 (+)
8779	Isolated Manchester 1953	n.d. (−)	n.d. (−)	n.d. (−)	18.50 (+)

n.d. not detected

showed a slight decrease in body weight (Fig. 1). All other mice looked healthy and were behaving normally, with no visible symptoms of disease. After 48 h upon infection, the mice infected with HmsF⁻ strains apparently were able to control the infection and gained body mass. However, the mice infected with the wild-type (i.e., positive for all tested markers) *Y. pestis* strains started to show a significant loss of body mass (Fig. 1) and appeared listless and hunched up. At 72 h post-infection, the mice that received the wild-type *Y. pestis* started to lose even more body mass and seemed totally unable to control the infection, whereas all mice infected with the mutant *Y. pestis* strains were behaving normally and showed no clinical signs of pneumonic plague.

Post-mortem analysis of tissues To investigate whether the bacterial clearance differed when one or more of the virulence markers were lacking, the bacterial loads in the
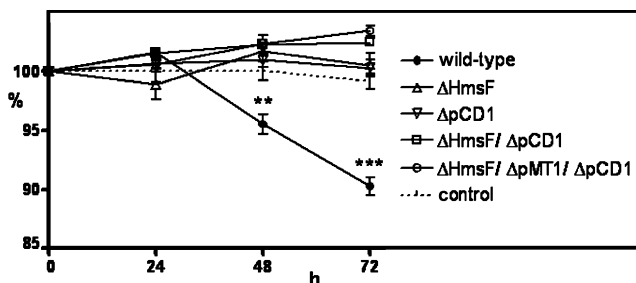

Fig. 1 Mice infected with the mutant *Yersinia pestis* strains show no decrease (in percent) in body mass (in percent). Balb/c mice were i.n. infected with 2×10^5 CFU wild-type or mutant *Y. pestis* and monitored for body mass. Results are expressed as means± SEM ($n=5$ per strain); significance was determined by two-way ANOVA (**$p<0.01$; ***$p<0.001$)

spleen, lung and liver were determined. In the organs of mice infected with *Y. pestis* strains containing all the investigated virulence genes (as analyzed by PCR), mean bacterial counts of approximately 10^6 CFU/mg lung tissue were found (Fig. 2a). However, in animals challenged with strains missing one or more virulence genes, the bacterial loads in the lungs were at least 4 logs lower than was observed with the fully virulent strains. Similar results were obtained when liver (Fig. 2c) and spleen tissue (Fig. 2b) were analyzed.

In addition, the mass of lung, spleen and liver tissues was determined post-mortem. Interestingly, the masses of the lungs from mice challenged with strains possessing a full complement of virulence genes was significantly higher than from mice challenged with strains missing one or more virulence genes (Fig. 3a). This was presumably due to the observed hemorrhage in tissues from mice challenged with virulent strains (Fig. 3b). Lungs from mice infected with the mutant strains uniformly exhibited lower lung masses.

In vitro analysis of the role of the Y. pestis virulence markers in cytotoxicity To determine the role of the virulence plasmids in *Y. pestis* induced cytotoxicity human PBMCs were isolated from three volunteers and cultured in the presence of the *Y. pestis* strains described in Table 2. After 48 h, cytotoxicity was determined by measuring the release of lactate dehydrogenase in the culture supernatant due to cell lysis. As expected the wild-type strains were highly toxic to the PBMCs. In contrast, a slight decrease in cytotoxicity was observed in the *Y. pestis* strains lacking pCD1 compared with the wild-type strains (Fig. 4), which is probably due to the absence of the TTSS that is encoded by the pCD1 plasmid. In the *Y. pestis* strains lacking the

Fig. 2 Decreased bacterial loads (log CFU/mL) in case one or more of the virulence markers is missing. Balb/c mice were i.n. infected with 2×10^5 CFU wild-type (*wt*) or mutant *Yersinia pestis* (*F* ΔHmsF, *1* ΔpCD1, *F1* ΔHmsF/ΔpCD1, *F12*, ΔHmsF/ΔpCD1/ΔpMT1, *C* control) and euthanized at 72 h post-infection. Numbers of CFU per organ in lung (**a**), spleen (**b**), and liver (**c**) were determined. Results are expressed as means±SEM (*n*=5 per strain); significance was determined by comparison of the mutant strains to the wild-type strain using Student's *t* test analysis (*$p<0.05$; **$p<0.01$; ***$p<0.001$)
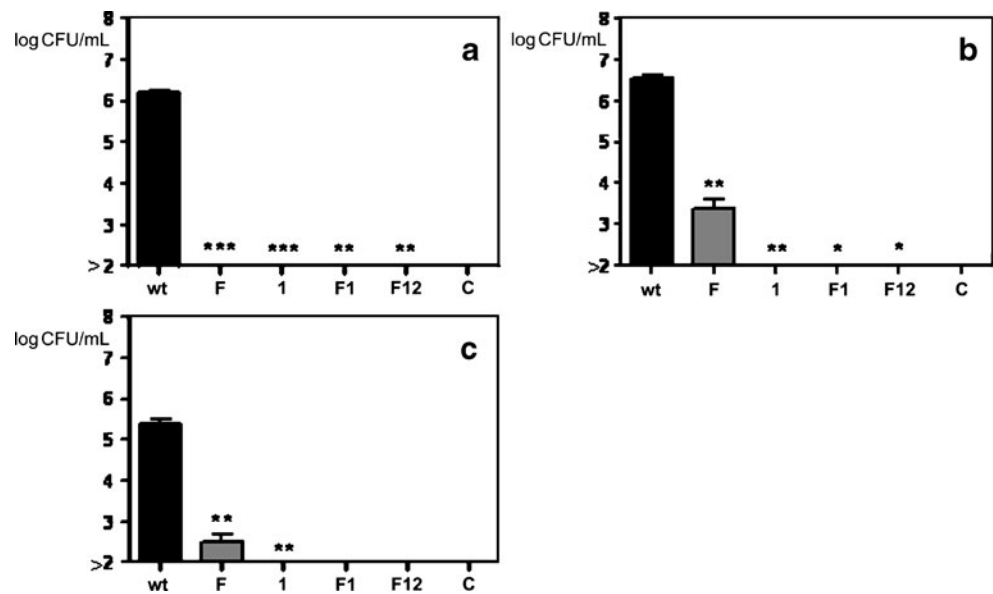
HmsF but possessing pCD1, no decrease in cytotoxicity was detected.

In vitro analysis of Y. pestis induced cytokine production The production of pro- and anti-inflammatory cytokines by immunomodulatory cells is a crucial component of the host response to control bacterial infection. To study the role of the *Y. pestis* virulence plasmids in the induction of cytokine production, human PBMCs were isolated from three volunteers and cultured in the presence of the *Y. pestis* strains described in Table 2. At 4 and 48 h post-infection, levels of TNF-α and IL-6 were measured in the culture supernatants using ELISA. A significant increase in cytokine production was observed in all pCD1⁻ strains (Fig. 5). The only exception was the *Y. pestis* strain negative for HmsF, pCD1, and pMT1, which showed an increase in IL-6 levels, but no increase in TNF-α production. Again, no differences were observed between the wild-type strains and the HmsF⁻ strains.

Discussion

This study set out to determine the relationship between the presence of a number of key *Y. pestis* genes and virulence as determined by the mouse model of pneumonic plague. The aim was to be able to use a simple molecular assay in order to predict virulence and hence outcome in the mouse model.

The binding by *Y. pestis* strains of the dye Congo red has been historically used as an indicator of the presence of a number of virulence traits related to iron uptake (Perry et al. 1990). The combination of the virulence factors collectively known as the Pgm⁺ (pigmentation) phenotype is used to describe the genetic linkage between the *hmsHFRS* locus required for Congo red binding (Crb⁺) and the virulence genes encoding a siderophore-based iron-uptake system (Perry et al. 1990); loss of this locus from *Y. pestis* occurs at high frequency (Brubaker 1969) and is the result of a 102 kb chromosomal deletion (Hare and McDonough
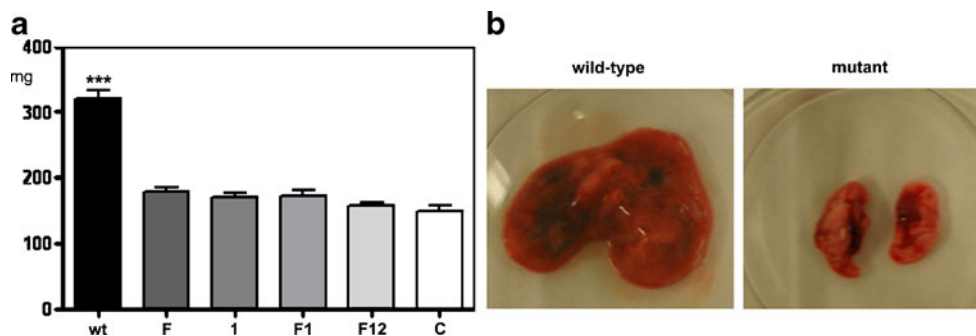

Fig. 3 Infection with wild-type *Yersinia pestis* leads to increased lung mass (milligrams) and hemorrhage in the tissues. Balb/c mice were i.n. infected with 2×10^5 CFU wild-type or mutant *Y. pestis*. At 72 h post-infection the animals were euthanized and lung mass was determined (**a**). An example of hemorrhage in the tissues is shown in (B). Results are expressed as means±SEM (*n*=5 per strain). Significance was determined by comparison of the lung masses of the infected animals to the uninfected, control animals using Student's *t* test analysis (***$p<0.001$); for abbreviations of strains, see Fig. 2
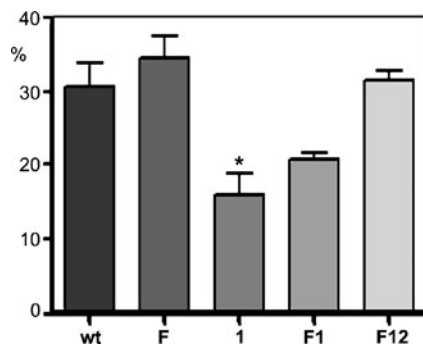
Fig. 4 Absence of the pCD1 plasmid leads to a slight decrease in cytotoxicity (in percent). Human PBMCs were infected with wild-type or mutant *Yersinia pestis* at a multiplicity of infection of 10. At 48 h post-infection the cytotoxicity was determined by measuring the lactate dehydrogenase activity. Data are representative of three independent experiments; results are expressed as means±SEM. Significance was determined by comparison of the mutant strains to the wild-type strain using Student's *t* test analysis (**$p<0.05$); for abbreviations of strains, see Fig. 2

1999). Congo red binding is only observed following growth at 26°C and absence of the *hmsHFRS* locus does not affect virulence in mammalian bubonic plague models (Perry et al. 2004). While the relationship between mice challenged via peripheral routes of infection and the presence of the Crb$^+$ phenotype is clear, no such relationship has been described for animals challenged via the intranasal route resulting in the pneumonic model of plague.

Our data indicate that pCD1 as well as HmsF are essential for virulence in the pneumonic plague model; the absence of one or more of these loci resulted in *Y. pestis* strains with decreased virulence. The precise role in virulence for pMT1 and pPCP1 is still unknown and no strain was available lacking only these two loci. When mice were challenged with strains possessing a full complement

of the loci examined, all resulted in clinical disease with concurrent manifestations of disease confirmed at post-mortem examination. During the study they exhibited ruffled fur, significant weight loss and appeared to be moribund at 72 h post-infection. All mice that received one of these fully virulent strains also exhibited increased lung hemorrhage with concomitant lung mass increase when compared with control groups. The mice which received any of these five strains also had much higher numbers of bacteria within the lung, spleen and liver tissues when compared with either uninfected control groups or to animals which received a mutant strain.

Animals challenged with HmsF$^-$ strains of *Y. pestis*, however, were not completely free of clinical symptoms of disease. These animals initially exhibited symptoms of disease, initially having ruffled fur and exhibiting body mass loss. These animals appeared to rapidly control the infection and by 48 h post-challenge, regained body mass and were not exhibiting signs of distress (no ruffled fur). These data indicate that, although initial infection appears to proceed at a similar rate to that with wild-type strains, the animals are able to overcome the infection relatively rapidly; this may be attributable to the loss of the HmsF phenotype and hence to the inability of these particular strains to sequester iron within the mouse macrophages.

The role for hemin- or CR-binding systems in host cell adherence, invasion and survival has been described for other pathogens (Daskaleros and Payne 1987; Garduno and Kay 1992; Stugard et al. 1989). Due to its ability to bind nitric oxide (NO), hemin can protect HmsF$^+$ *Y. pestis* cells from its bactericidal effects (Morris et al. 1995; Vazquez-Torres et al. 2008). This thesis is reinforced by results of Lillard et al. (1999), who described that the addition of hemin to HmsF$^+$ cells can led to an increased protection against the NO donor NOR-1. Thus in *Y. pestis* strains
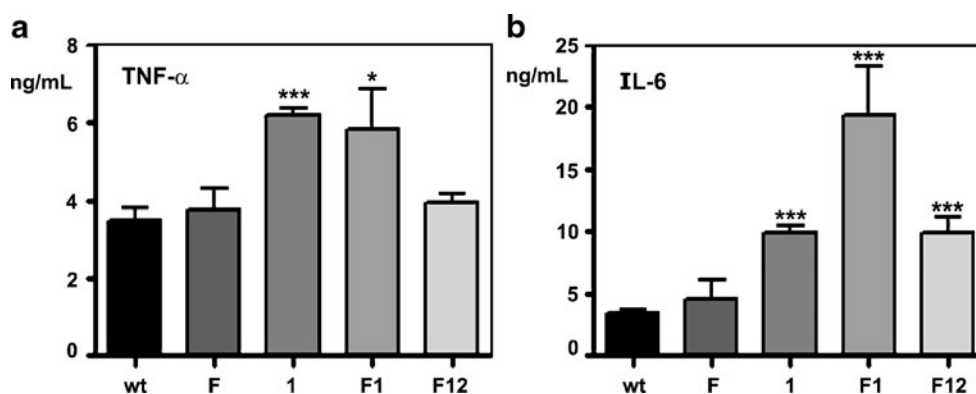

Fig. 5 TNF-α and IL-6 production (both in nanograms per milliliter) is altered by pCD1. Human PBMCs were infected with wild-type or mutant *Y. pestis* at a multiplicity of infection of 10. At 4 and 48 h post-infection, respectively, TNF-α (**a**) and IL-6 (**b**) culture supernatant levels were determined by ELISA. Data are representative of three independent experiments; results are expressed as means±SEM. Significance was determined by comparison of the mutant strains to the wild-type strain using Student's *t* test analysis (*$p<0.05$; ***$p<0.001$); for abbreviations of strains, see Fig. 2

lacking HmsF the antimicrobial function of NO is not inhibited by hemin. This may be an explanation for the increased bacterial clearance that was observed following infection with the HmsF⁻ strains compared with the wild-type *Y. pestis*. Moreover, no changes in cytokine levels were detected after infection with HmsF⁻ *Y. pestis* strains when compared with the wild-type strains. In contrast to the pCD1 plasmid, HmsF seems to have no function in the inhibition of cytokine production.

To survive inside the host and maintain a persistent infection, *Y. pestis* uses a variety of mechanisms to evade the host immune response (Li and Yang 2008). One of those mechanisms is encoded by the pCD1 plasmid, namely the Type III Secretion System (TTSS) (Viboud and Bliska 2005). *Y. pestis* uses the TTSS to inject the *Yersinia* outer proteins (Yops) into macrophages, dendritic cells, and neutrophils to inhibit the responses of the host immune system (Cornelis 2002; Juris et al. 2002; Navarro et al. 2005). One of the key players in the TTSS is the pCD1 encoded LcrV protein (Smiley 2008). Besides its prominent role in delivering the Yops into the cell, LcrV is exposed on the bacterial cell surface to play a role in cell-cell adhesion (Holmstrom et al. 1997). After being secreted into the environment, LcrV exploits TLR2 and CD14 to trigger the release of IL-10 by the host immune cells to suppress the production of pro-inflammatory cytokines, such as TNF-α and IFN-γ (Sing et al. 2003). This important role for pCD1 (or more specifically the TTSS), in inhibiting the host immune response and inducing cytotoxicity is confirmed by our results. PBMCs exposed to *Y. pestis* strains lacking the pCD1 plasmid, and thus the TTSS, showed increased cytokine levels and a slight decrease in cytotoxicity compared with the wild-type strains. The only exception is the *Y. pestis* strain which, besides pCD1, also lacks the HmsF and pMT1 markers; no increase in TNF-α levels was observed. Probably this unaltered TNF-α production is due to the absence of the Fraction 1 (F1) protein which is encoded by the pMT1 plasmid. DCs stimulated with *Salmonella typhi*-expressing F1 strain primed strong Ag-specific CD4+ and CD8+ T cells (Ramirez et al. 2009). These cells secrete phagocyte-activating cytokines, such as IFN-γ and TNF-α (Kaufmann 1993). This role for F1 in TNF-α production was confirmed by Sodhi et al. (2004) who showed that macrophages stimulated with rF1 for 24 h produce high TNF-α levels. The absence of both the cytokine suppressing pCD1 as well as the cytokine stimulating pMT1 may be the explanation of the unaltered TNF-α levels that we observed in vitro. However, after infection with strains lacking pCD1, HmsF and pMT1, a significant increase in IL-6 level was observed. This suggests that the pMT1 plasmid (probably its product the F1 antigen), stimulates the production of TNF-α without affecting the expression of the IL-6 cytokine. The precise

function of the pMT1 plasmid in the above process remains unknown as no *Y. pestis* strain lacking the pMT1 plasmid was available during this study. Clearly, this comparative in vivo virulence study provides novel insights into the role of HmsF in pneumonic plague.

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